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Quantitative Analysis of Wide-Field Specular Microscopy

II. Precision of Sampling from the Central Corneal Endothelium

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The precision of the measurement of mean endothelial cell area obtained by sampling with small-field and wide-field specular microscopy from the central 4 mm of human corneal endothelium was studied by comparing endothelial cell parameters from individual specular micrographs in vivo to the results obtained by montaging the micrographs from the entire central 4 mm of the same corneas. The small samples were at least 10% from the true mean cell size of all cells of the central 4 mm in any endothelium other than that with the most homogeneous pattern. A new algorithm for sampling with these two specular microscopes will need to be derived to permit a more precise measure of the mean area of endothelial cells in the central 4 mm of the human corneal endothelium. Invest Ophthalmol Vis Sci 30:1972–1979, 1989

The long-term health and clarity of the cornea is critically dependent upon the maintenance of a functional endothelial layer. The most common cause of damage to this layer is as a result of intraocular surgery, including cataract extraction, intraocular lens implantation and penetrating keratoplasty. The only clinically available methods of investigation of this layer are currently: (1) pachometry; (2) fluorophotometry; and (3) specular microscopy. Specular microscopy has become the mainstay for the assessment of patients prior to surgery[1–4] and as an investigative tool to evaluate new surgical procedures and their effect on the corneal endothelium. The quantization of endothelial cells by this method, in current and past clinical studies, has relied upon small samples of endothelial cells in the central 4 mm of the cornea.[1–4] The reliability of this method of sampling in endothelial layers with any polymegathism of cell sizes and shapes has been seriously questioned.[5,6] An initial study highlighted the inaccuracies of the counting or assessment of samples taken by small-field specular microscopy[6]; these samples do not provide an accurate representation of the surrounding 1 mm^2 of corneal endothelium in anything other than the most regular endothelial mosaic. Similarly for wide-field specular microscopy, sampling less than 20% of the entire specular micrograph provided an inaccurate representation of the full specular micrograph in anything other than the most regular of endothelial mosaics.

We wish to extend this study[6] to examine the adequacy of sampling by both small-field and wide-field specular microscopy in an area of approximately 4 mm of central corneal endothelium in the human.

Materials and Methods

Contact specular microscopy using a Keeler-Konan wide-field specular microscope (Osaka, Japan) was performed on a series of 11 human corneas, where the endothelial state ranged from extremely regular and homogeneous through to extreme polymegathism in size and shape. Full informed consent was obtained from the volunteers. The photographic method included repetitive overlapping photographs performed while moving the applanation cone slowly up and down the cornea, completing a vertically oriented grid pattern and extending out to the posterior corneal rings in all areas (approximately 4 mm in diameter). Approximately 72 exposures were taken on each cornea (Fig. 1).

Prints measuring 12.5 × 17.5 cm were made from the resultant negatives and the appropriate prints cut and pasted into a photographic montage of the central corneal endothelium. A similar montage was

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then made by using the appropriate negatives enlarged to 27.5 × 35 cm prints.

This photographic montage was then artificially divided into adjacent rectangular areas, each of which was the equivalent of one wide-field specular microscopic photograph (826 × 1212 μm or a field of approximately 3000 cells if endothelial density is normal). An acetate grid divided into 25 equal rectangles (each of which approximated one small-field specular micrograph, ie, 165 × 242 μm or a field of approximately 120 cells if the endothelial density is normal) was placed over each of these areas and a manual pen digitizer used to trace the outline of all visible cells in the central corneal endothelium (Fig. 2). The data were collected per single rectangle per wide-field specular microscopic area by including cells that crossed the left and superior boundaries of each of the individual, small rectangles, together with those entirely within the small rectangles. The cell areas from all rectangles were pooled to obtain the "true" population mean cell area (or density) and the population standard deviation for each central corneal endothelial montage.

In an effort to evaluate the precision of current sampling techniques used to estimate the mean cell area (or cell density) and population standard deviation, samples of varying sizes and various configurations were taken from each montage to allow different sampling techniques to be compared. Basically, two different sampling formats were used; they will be referred to as "fixed" sampling and "random" sampling.

Fixed Sampling

Each montage consisted of between 20 and 42 artificially defined wide-field specular micrographs (WFSM) as described above. Each of these contrived WFSMs were considered an individual sample from the central 4 mm of corneal endothelium. For each WFSM, a mean cell area and standard deviation was derived for that WFSM, and this sample mean was compared to the "true" population mean of the entire central 4 mm of cornea using the parameter of percent error of each sample mean from the true mean. It was then calculated how many of these sample means fell within 10% of the true population mean.

The next step of the evaluation of the central corneal endothelium consisted of repeating the above comparison, using the nine central small rectangles (each rectangle being equivalent to one small-field specular micrograph) in each WFSM (Fig. 3) instead of all 25 rectangles as the sample. Once again, it was calculated how many of the smaller sample means fell within 10% of the true population mean.

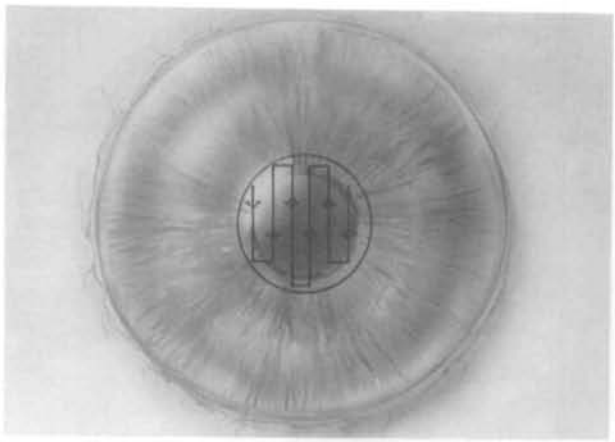

Fig. 1. Schema of in vivo "montage" photography with wide-field specular microscope covering the central 4 mm of the cornea.

The above method was repeated, using a sample mean taken from five central small rectangles (each rectangle being equivalent to one small-field specular micrograph) of each WFSM, then three small rectan-

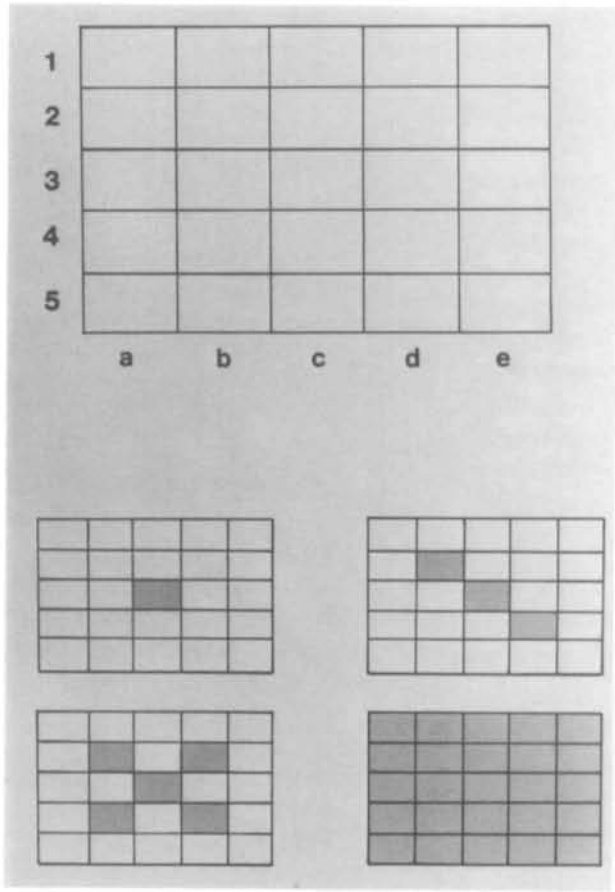

Fig. 2. Representation of acetate grid used as overlay for digitizing wide-field micrograph (above) and the individual small rectangles (equivalent to one small-field specular micrograph each) sampling techniques from each of the wide-field specular micrographs (below).

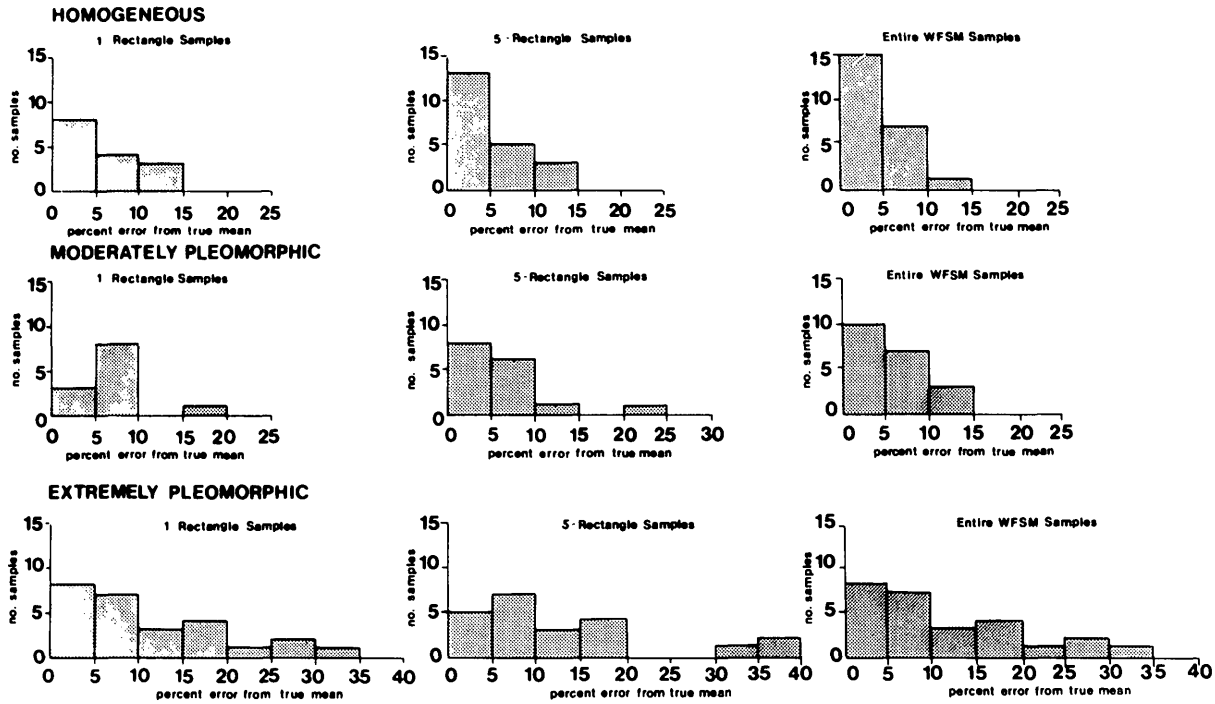

Fig. 3. Percentage error from the true mean of varying number of small rectangle samples obtained from the entire central 4 mm of cornea in varying degrees of endothelial polymegathism.

gles, and finally the one central rectangle (Fig. 2). These sample means were also compared to the overall 4 mm of central corneal endothelium in a similar manner.

As an alternative method of evaluating the accuracy of these different sampling schemes, 95% confidence intervals were calculated for the percent error from the true mean. These confidence intervals give a range about the true mean within which one can generally expect the mean of a sample of a given size to lie. The samples also were assessed for their ability to estimate the population standard deviation. The proportion of samples that underestimated the true standard deviation by more than 10% was computed for the various sample sizes.

Random Sampling

In this sampling scheme, the same sample sizes were drawn as for the fixed samples, but the specified number of rectangles were randomly chosen from anywhere in the montage, not fixed to the center of each WFSM. Two hundred random samples of a single rectangle were drawn, with each of these creating a sample. This was repeated for sample sizes of three, five, nine and 25 rectangles. In addition to these sampling schemes, ten cells were randomly drawn from the entire montage, to create a sample, and this was repeated 100 times. This was also repeated for randomly selecting 20, 50, 100 and 200 cells from the entire montage.

The accuracy of these samples was measured as for the fixed sampling scheme. Each sample size was evaluated to show what percentage of the samples gave a mean within 10% of the true population mean along with 95% confidence intervals of the percent error around the true population mean. In addition to this, the number of samples that underestimated the population standard deviation by more than 10% was computed for the samples of various numbers of randomly selected cells.

Results

Between 5000 and 28,000 cells were digitized for each corneal endothelial montage by decreasing cell density (Table 1). The montages with the most regular mosaic were those in which the most cells could be visualized and digitized. The characteristics of the endothelial cell populations in the 11 montages are seen in Table 1.

Figure 3 illustrates the frequency distribution of percent error from the true mean cell area by taking fixed samples of one small rectangle (each rectangle being equivalent to one small-field specular microscopy), five small rectangles and entire wide-field specular micrographs from a montage of homogeneous endothelium, one of moderate polymegathism

Table 1. Descriptive statistics of central corneal endothelial cell populations

Montage number and description		*Number of cells*	*Cell density**	*Mean cell area†*	*SD†*	*CV‡*	*Min†*	*Max†*
Homogenous	1	25,416	3894	257	75	29%	40	884
	2	28,048	3660	273	95	34%	50	1233
Moderate polymegathism	3	18,912	3660	314	98	31%	48	937
	4	23,036	3080	325	119	37%	23	1048
	5	20,162	2886	346	114	33%	47	1506
	6	19,214	2779	360	119	33%	48	1620
Marked polymegathism	7	14,490	2534	395	328	83%	41	16,074
	8	9170	1739	575	243	42%	48	2055
	9	10,309	1312	762	285	37%	48	3162
	10	5266	963	1039	684	66%	48	5410
	11	8925	818	1223	391	32%	163	4200

* Cells/square millimeters.
† Square microns.
‡ Coefficient of variation = SD/mean × 100.

and one of extreme polymegathism. Although sampling from a regular endothelial mosaic by one, five or 25 rectangles provides an accurate representation within 15% of the true mean area of the central corneal endothelium, as the endothelium becomes more irregular, the accuracy of samples of even the size of an entire wide-field specular micrograph diminishes greatly.

In Table 2, the accuracy of selecting "fixed" samples of sizes one, three, five and nine rectangles, and an entire WFSM from the montages of central corneal endothelium is tabulated. The Table shows what percentage of sample means fell within 10% of the true population mean for each sample size. Although sampling from a regular endothelial mosaic by one, three, five, nine or 25 rectangles provides an accurate representation of the true mean about 80% of the time, as the endothelium becomes more irregular, the accuracy of samples of even the size of an entire WFSM diminishes greatly.

The confidence intervals show that one can be reasonably assured of getting a sample mean that is within 20% of the true mean for fixed samples of all sizes of homogeneous montages and montages with moderate polymegathism (Table 3). However, as the polymegathism of the endothelium in the montage becomes greater, the confidence interval widens, indicating that the sample may be as far off as 30–60% from the true mean. Table 4 shows the percentage of samples that underestimated the population standard deviation by more than 10%. Generally, for the montages with homogenous and montages with moderate polymegathism, taking a larger size sample improved the estimate. However, in the montages with marked polymegathism, 50–90% of the samples underestimated the standard deviation by more than 10%, regardless of sample size.

Table 5 shows the percentage of sample means that were within 10% of the true mean, for each sample size in the random sampling scheme. The more rec-

Table 2. Fixed sampling: percent of samples within 10% of population mean

Montage number and description		*Number of WFSM*	*Mean cell area**	*Number of rectangles†*				*Entire WFSM (%)*
				1 (%)	*3 (%)*	*5 (%)*	*9 (%)*	
Homogenous	1	25	257	73	63	76	76	92
	2	24	273	71	70	90	86	92
Moderate polymegathism	3	24	314	86	76	88	71	83
	4	27	325	59	91	74	74	78
	5	27	346	43	55	64	56	63
	6	20	360	47	81	94	100	95
Marked polymegathism	7	31	395	54	57	50	54	48
	8	22	575	21	21	30	35	23
	9	23	762	29	43	48	48	43
	10	41	1039	36	39	27	27	27
	11	21	1223	87	69	63	69	76

* Square microns.
† Each is equivalent to one small-field specular micrograph.

Table 3. Fixed sampling: 95% confidence interval for percent error from population mean

Montage number and description		*Mean cell area**	*Number of SF rectangles‡*				
			1	*3*	*5*	*9*	*Entire WFSM*
Homogenous	1	257	20†	22†	20†	16†	12†
	2	273	17	16	15	15	15
Moderate polymegathism	3	314	18	18	17	17	15
	4	325	21	14	17	19	18
	5	346	31	26	24	25	18
	6	360	25	14	12	10	11
Marked polymegathism	7	395	47	45	31	34	31
	8	575	62	59	55	55	57
	9	762	46	47	47	47	47
	10	1039	43	42	61	58	44
	11	1223	12	28	27	22	17

* Square microns.
† Units = ± %.
‡ SF = small field.

tangles in the sample, the more likely it is to be within 10% of the population mean. Also, sampling from a regular endothelial mosaic gives means almost universally within 10% of the truth, while as the polymegathism of the endothelial layer becomes more marked, the accuracy decreases.

Selecting ten random cells from the entire montage resulted in sample means surprisingly close to the true mean, and again the accuracy increases as the sample gets larger, and decreases with increasing polymegathism of the endothelial layer.

Table 6 shows the same trends as the other tables. In considering an endothelium with polymegathism, a sample of five or nine rectangles is likely to give a mean that is within 15–30% of the true population mean. In samples of randomly selected cells, the larger samples gave better estimates of the standard deviation, especially in the more homogenous mosaics, but generally the samples tend to underestimate the true standard deviation, except where 200 random cells were selected.

Discussion

Specular microscopy has been widely used over the last decade to analyze human and experimental animal corneal endothelial densities[7] and other parameters to establish baselines for scientific studies or preoperative values for individual patients. Numerous third-party medical insurance companies in the USA now reimburse for specular microscopy as part of preoperative patient care, presumably on the basis that the methodology currently used enables the physician to obtain an accurate estimate of the individual's corneal endothelial density or other parameters.

Multiple federally funded grants in the USA have

Table 4. Fixed sampling: percent of samples that underestimate standard deviation by more than 10%

Montage number and description		*Number of WFSM*	*SD**	*Number of rectangles*				*Entire WFSM (%)*
				1 (%)	*3 (%)*	*5 (%)*	*9 (%)*	
Homogenous	1	25	75	36	30	21	17	8
	2	24	95	24	30	14	14	4
Moderate polymegathism	3	24	98	36	29	24	18	17
	4	27	119	47	32	30	17	15
	5	27	114	57	32	32	33	26
	6	20	119	47	25	25	19	15
Marked polymegathism	7	31	328	96	89	82	75	71
	8	22	243	80	89	90	90	82
	9	23	285	71	71	71	71	52
	10	41	684	90	81	63	72	68
	11	21	891	40	50	50	38	24

* = Square microns.

Table 5. Random sampling: percent of samples within 10% of population mean

Montage number and description		Mean cell area*	Random small-field rectangles					Random cells				
			1	*3*	*5*	*9*	*25*	*10*	*20*	*50*	*100*	*200*
Homogenous	1	257	79†	100†	100†	100†	100†	70†	92†	97†	100†	100†
	2	273	68	91	98	99	100	57	77	95	100	100
Moderate polymegathism	3	314	69	97	99	99	100	68	79	98	100	100
	4	325	76	96	100	100	100	66	78	95	100	100
	5	346	58	89	95	99	100	71	89	94	97	100
	6	360	72	92	99	100	100	69	89	87	92	99
Polymegathism	7	395	41	57	69	78	96	33	52	62	72	77
	8	575	34	52	62	74	95	60	78	81	91	95
	9	762	39	65	79	91	100	63	86	88	92	100
	10	1039	27	52	59	74	98	36	32	66	73	84
	11	1223	72	93	98	100	100	74	87	83	90	96

* Square microns.

† Units = %.

also used as their baseline the ability of the specular microscope to sample adequately the human and experimental animal corneal endothelium. Over 100 articles have been published in the scientific journals using small-field and wide-field specular microscopy in an attempt to generate data and statistics about the adequacy or superiority of various surgical procedures or intraocular solutions and devices. To be able to do so accurately, large enough groups of patients would be needed to compensate for the high variability demonstrated by current sampling techniques in the individual patient.

A number of studies have attempted to address the adequacy of sampling by small-field and wide-field specular microscopy from the central corneal endothelium, with varying results.[6,8-11] None of these studies have attempted to correlate the true central endothelial parameters with those obtained by the usual sample sizes from wide-field and small-field specular microscopy. Our study shows the feasibility of photographically montaging the central corneal endothelium and obtaining an accurate true population mean of approximately the central 4 mm of corneal endothelium. It demonstrates the difficulty in obtaining reliable information (within 10% of the true mean) by using small-field specular microscopy (equivalent of one rectangle) in endothelium of even the most regular mosaics (Fig. 4). However, even counting an entire wide-field specular micrograph (not a usual practice) does not permit a reliable estimate of the central corneal endothelium within 10% of the true mean or standard deviation (Fig. 5).

Many researchers now recognize that other parameters of corneal endothelium cells such as form factor and hexagonality[12-15] may represent a more accurate measurement of an individual's endothelium as photographed by a specular microscope than cell density measurements. Yet cell counting or mean cell area

Table 6. Random sampling: 95% confidence interval for percent error from population mean

Montage number and description		Mean cell area*	Random cells					Random small-field rectangles				
			1	*3*	*5*	*9*	*25*	*10*	*20*	*50*	*100*	*200*
Homogenous	1	257	15†	9†	8†	5†	3†	18†	13†	9†	6†	4†
	2	273	20	12	9	6	4	23	15	10	7	5
Moderately pleomorphic	3	314	20	12	9	6	4	23	15	9	7	5
	4	325	18	10	8	6	4	22	17	10	8	6
	5	346	27	13	10	7	5	19	12	9	7	4
	6	360	19	12	9	6	4	20	13	9	6	4
Pleomorphic	7	395	38	27	23	17	10	44	34	30	19	12
	8	575	51	30	23	17	10	25	17	12	9	6
	9	762	41	22	18	11	7	23	15	10	7	5
	10	1039	57	30	24	18	8	37	45	18	13	9
	11	1223	22	11	9	7	4	19	13	9	7	5

* Square microns.

† Units = ± %.

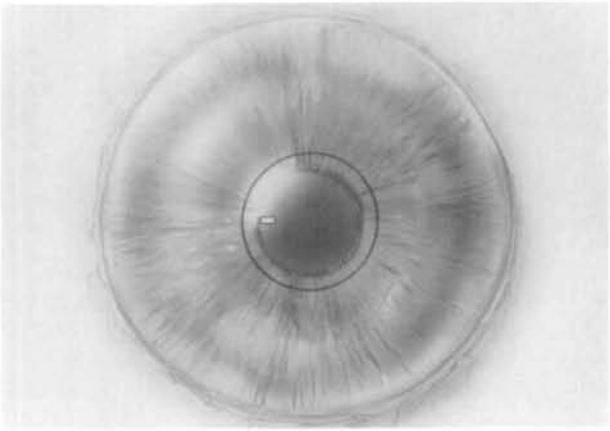

Fig. 4. To scale representation of one small-field specular micrograph in the central 4 mm of cornea.

measurements remain the mainstay of the clinician's evaluation of specular micrographs before and after surgery.

In past studies and in current research, the number of cells counted is usually between 50 and 300.[1,2] These are usually counted within a single area, or perhaps in two or three scattered areas of the central corneal endothelium. The use of specular microscopy as a preoperative evaluative tool in patients is most relevant and commonly practiced in those patients who already have had surgery and have an endothelium with moderate to marked polymegathism. The above sample sizes are unacceptably inaccurate in evaluating the central endothelial density and other parameters.

Comparison of the same sample size between Tables 2 and 5 show that the "random" samples give better estimates of the true mean, despite the fact the same number of rectangles were sampled. This is an indication of a "clustering" effect; that is, there must be clusters of small cells and clusters of large cells, so that by simple random sampling from all over the montage, as opposed to from the one WFSM, one gets a better representation of what the central 4 mm of corneal endothelium is like.

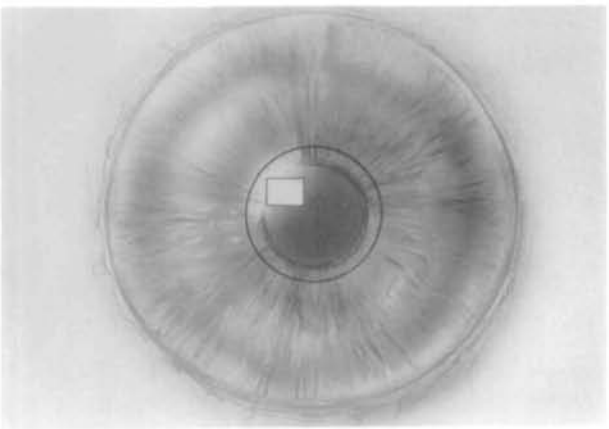

Fig. 5. To scale representation of one wide-field specular micrograph in the central 4 mm of cornea.

Similar comparisons can be made between Tables 3 and 6.

Likewise, the relatively small samples of ten, 20, 50, 100 and 200 random cells gave surprisingly precise results, again attesting to the clustering theory. If we were able to randomly select 20 cells or 50 cells from anywhere in the central corneal endothelium, amazingly good results would be found except in the endothelial layer with severe polymegathism.

Although simple random sampling of between ten and 200 cells from the entire montage would appear to be an excellent method of evaluating the central corneal endothelium, it is unfortunately not practical because of the great investment of time and money in obtaining a complete montage of the central 4 mm of corneal endothelium. The high percentage of samples that underestimate the standard deviation is more evidence of a clustering effect. In fact, there were actually relatively few samples that overestimated the standard deviation by 10% or more. This underestimation has important implications when sample data are used to compute confidence intervals (which will in turn be underestimated) or to detect the changes in an endothelium over a period of time. Because of the poor estimate of variability, the ability to correctly identify a change in mean cell area is greatly reduced.

In a previous study, we have shown the inadequacy of the current methods of sampling within one wide-field specular micrograph.[6] Our current study extends this problematic sampling to the central 4 mm of corneal endothelium. It is evident that an algorithm needs to be developed which will permit the investigator to sample sufficient endothelial cells from the central corneal endothelium to give a reasonably reliable prediction of the true mean and other parameters of the central corneal endothelium. The broader question that addresses the relationship between the mean cell area (density) and standard deviation of these central corneal endothelial cells and the endothelial cells peripheral to this area,[16] still requires further investigation.

Key words: specular microscopy, corneal endothelium, sampling, precision, cell area

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